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IDENTIFICATION OF CUCUMBER DAMPING-OFF BASED ON MORPHOLOGICAL AND MOLECULAR CHARACTERIZATIONS IN RAFSANJAN*

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Abstract

Cucumber damping-off is a limiting factor in commercial greenhouse production. To determine the causal agents of disease, sampling and fungal isolation were performed during 2009 and 2011. A total of 23 isolates of *Pythium* sp. were obtained using selective media. Their pathogenicity was evaluated on cucumber seedlings under greenhouse conditions. All isolates were pathogenic. Morphological and molecular identifications of the isolates were performed. *P. aphanidermatum* was identified based on microscopic characteristics. The complete sequences of ribosomal DNA internal transcribed spacers regions of selected isolates were determined and submitted to GenBank. The GenBank-BLAST homology search revealed *P. aphanidermatum* as the most similar sequence (> 99% identity) with GenBank entry AB355599. PCR primers were designed for the identification of *P. aphanidermatum* in which had a unique ITS sequence. The PCR primer combination Paph54F/ITS2 was species-specific with no cross-reaction to other *Pythium* species or to soil borne cucumber fungal pathogens tested. The detection limit was as little as 200 fg pure genomic DNA. *P. aphanidermatum* could be detected in lesions of infected cucumber stem tissue. No signals were obtained from healthy cucumber tissue DNA.

Keywords: DNA extraction, Sequencing, ITS, Conventional PCR, Detection.

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Introduction

Cucumber is one of the most important greenhouse product which is annually grown widely. Continuous cultivation of this plant caused contamination to several soil-inhabiting pathogens. Damping-off, as the most seedling root and crown rot disease, is a limiting factor in cucumber greenhouse cultivation (Shirazifard & Banihashemi 2007). Losses up to 25 percent were reported under optimal conditions yearly (Etebarian 2002). *Pythium* spp. are most often responsible for damping-off, but several other pathogens, including *Rhizoctonia* sp., *Fusarium* spp., and *Phytophthora* spp., can also cause decay. Successful control of the disease depends on accurate identification of pathogen. Different species of *Pythium* has been reported on cucumber (Favrin *et al.* 1988; Nasrollah-Nezhad *et al.* 1998; Safarnezhad 2004; Chehri *et al.* 2010; Zamanizadeh *et al.* 2011). Historically, keys for identification of *Pythium* species are based on microscopic morphometrics and growth characteristics on specific media as well as biological characters (Van der Plaats-Niterink 1981; Mostowfizadeh-Ghalamfarsa & Banihashemi 2005). However, the overlapping of characters used for species classification frequently makes identification difficult and time intensive. Therefore alternative approaches must be developed to accurately identify and differentiate fungal species. DNA sequence analysis offers a less ambiguous way to determine *Pythium* species identity (Lévesque & de Cock 2004; Bala *et al.* 2010; Long *et al.* 2010; Long *et al.* 2011). Recently many molecular approaches including polymerase chain reaction (PCR) has been tested to identify *Pythium* spp., (Tambong *et al.* 2006; Klemsdal *et al.* 2008) as well as other plant pathogenic fungi (Alaei *et al.* 2009; Langrell *et al.* 2011). Species-specific molecular probes are a powerful means for detecting *Pythium* in soil and plant samples. DNA probes for several species of *Pythium* have been reported. Levesque *et al.*(1994) suggested the use of the internal transcribed spacer 1 (ITS1) region of the nuclear ribosomal DNA as a species-specific probe for *P. ultimum, P. aphanidermatum, Pythium acanthicum* Drechsler and *Phytophthora cinnamomi* Rands. Specific PCR primers of the ITS rDNA were used to identify five different *Pythium* species (Klemsdal *et al.* 2008) According to the high potential for loss from damping-off due to *P. aphanidermatum* a rapid detection and accurate identification of disease can be effective for disease management and control. Therefore the aim of this study was (i) to identify pathogens associated with cucumber damping-off based on morphological features and molecular analysis (ii) to develop a species-specific PCR primer to rapid detection of pathogen from plant tissue.

Materials and methods

Sampling and morphological identification of isolates

Sampling was performed from commercial cucumber greenhouses in Rafsanjan. Infected crown and roots were washed, surface-sterilized and plated on 1.5% water agar (WA) and corn meal agar (CMA) amended with 250 mg ampicillin, 50 mg benomyl and 10 mg rifampicin. The plates were incubated at 25 ± 1 °C. Baiting techniques with citrus leaflets (Mostowfizadeh-Ghalamfarsa & Banihashemi 2005) were used for isolation from soil samples. Isolates were morphologically compared with keys and descriptions of known *Pythium* species (Van der Plaats-Niterink 1981; Mostowfizadeh-Ghalamfarsa & Banihashemi 2005)

DNA extraction

Each isolate was grown on 100 ml of liquid corn meal medium. Genomic DNA was extracted from the pulverized mycelium using a modification of the cetyltrimethylammonium bromide (CTAB) extraction procedure described by Alaei *et al.* (2009). DNA of infected and non-infected cucumber stem tissue was extracted using 100 mg of stem tissue that was previously ground in liquid nitrogen with CTAB extraction procedure as described above.

Primer development, sensitivity and specificity

To develop candidate specific primers for *P. aphanidermatum* we aligned the rDNA ITS sequences of *P. aphanidermatum* isolates with the closely ITS sequence data from other *Pythium* species submitted to GenBank and *Cucumis sativus* (EF093522) using ClustalX 1.81 (Thompson *et al.* 1997). Regions that were conserved within the *P. aphanidermatum* isolates but differed from non-target organisms were used to develop candidate specific primers for *P. aphanidermatum*. In preliminary work for conventional PCR, one

candidate specific forward primer was designed manually, based on visual inspection of the multiple alignments. Candidate primer in combination with ITS2 (White *et al.* 1990) were checked using Primer 3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were synthesized by SPL company (SPL, Life Sciences, Republic of Korea). Primers were subjected to specificity tests using a selection of cucumber soil-borne pathogenic fungi as well as the sequences registered in GenBank using the standard nucleotide-nucleotide primer BLAST protocol (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences of ITS regions from other cucumber soil-borne pathogenic fungi were also aligned against the *P. aphanidermatum* sequence to ensure that the primer designed would be specific to the target pathogen. To assess the sensitivity of the detection of *P. aphanidermatum* using the designed primer, DNA dilution series resulting in two ng to 200 ag genomic DNA from isolate VRUC per PCR reaction were subjected to conventional PCR.

PCR amplification

PCR amplification of the rDNA ITS1-5.8S-ITS2 region was achieved with a PCR system C-1000 (Bio Rad, USA) thermocycler. PCR amplification was performed using the forward primer ITS6 (Cooke *et al.* 2000) and the reverse primer ITS4 (White *et al.* 1990). The reaction mixture and cycling parameters were modified as described by Cooke *et al.* (2000). Reagents were from GeNet Bio (South Korea) unless listed otherwise. The PCR cycle parameters consisted of an initial preheat at 94 °C (five min.) and 40 cycles, each consisting of one min denaturation at 94 °C, annealing for one min at 55 °C, and one min. extension at 72 °C. The reaction was ended with a final extension at 72 °C for 10 min. Five µl of PCR product were analyzed by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. MilliQ water was used instead of DNA template in the negative control.

For conventional PCR based DNA detection, primer combination forward Paph54F: 5'-CCGTTGAAATCATGTTCTGTGCTCTCTT-3'(This study) and reverse ITS2(White *et al.* 1990) was used. The reaction mix (50 µl) consisted of PCR Buffer (10 mM Tris–HCl, 50 mM KCl pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Roche), 0.2 µM of each primer, 1.25 units AmpliTaq DNA polymerase, and DNA template (in five µl) from target or non-target organisms, tested at different concentrations. MilliQ water was used instead of DNA template in the negative control. Annealing temperature (60 to 69 °C), annealing time (30 to 60 s), and extension time (45 to 60 s) were optimized for the primer set during preliminary experiments. Finally, the PCR cycles was performed as described above except for annealing at 67 °C for one min.

Sequencing of PCR product

PCR products were cleaned using the Accuprep® PCR purification Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Direct sequencing of the PCR products was done by the DNA sequencing laboratory of MilleGen (Labege, France). The complete rDNA ITS regions were sequenced in both directions using the primers ITS6 and ITS4. Sequence data were analysed using Chromas 1.45 (copyright ©1996-1998, Conor McCarty) and verified manually. DNA sequence data obtained in this study has been deposited in GenBank. DNA sequences were compared to homologous sequences registered in GenBank using the standard nucleotide-nucleotide BLAST protocol (http://www.ncbi.nlm.nih.gov/BLAST/). In order to determine the extent and location of ITS sequence variation between different isolates, the sequences were aligned using ClustalX 1.81(Thompson *et al.* 1997).

Result

Sampling and morphological identification of isolates

A total of 23 isolates were isolated from infected soil and root and crown rot of cucumber. After two days culture of infected tissue, aseptate hyphae grew on the plate very fast, forming white colonies with loose and aerial mycelia (results not shown). Based on the morphological measurements and growth response to temperature our isolates were identical to those of *Pythium aphanidermatum* (Edson) Fitzpatrick reported by Van der Plaats-Niterink (1981) and Mostofizadeh-Ghalamfarsa and Banihashemi (2005).

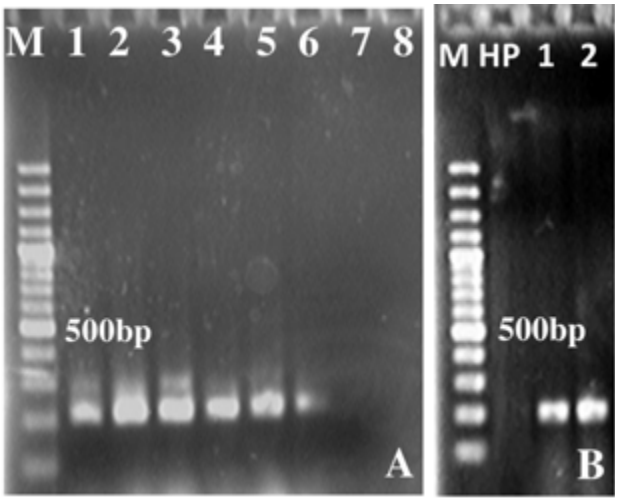

Fig. 1. Typical amplification products of conventional PCR with primer set PaphF54/ITS2. (A) Lanes 1 through 8 results from PCR reaction with a 10-fold dilution series of genomic *Pythium aphanidermatum* DNA as template, starting with 2 ng per reaction. (B) Lanes 1 and 2 results from PCR reaction with pure genomic DNA from *Pythium aphanidermatum* and infected plant tissue, respectively. HP=Cucumber healthy plant DNA. M=100-bp DNA ladder (Invitrogen).

Amplification and characterization of the rDNA ITS sequences

PCR amplification of the rDNA ITS region of *Pythium* isolates using the ITS6-ITS4 primer pairs was successful and gave PCR products of 900 base pair (bp) (results not shown). The complete genomic DNA sequences between the primers ITS6-ITS4 was obtained for the VRU and Lahijan isolates. The rDNA ITS nucleotide sequences of those isolates showed high sequence homology (100% identity). No significant intraspecific variation was observed. The sequences were submitted to GenBank. Their accession codes are listed as JF775593 and JF775594. The GenBank-BLAST homology search using these ITS sequences revealed *P. aphanidermatum* as the most similar sequence (99% identity) with GenBank entries AB355599, which is a partial and complete sequence of ribosomal RNA genes derived from vegetable soils (Kobayashi & Tojo 2008). The length of the PCR fragments in our study was 861 bp for ITS6-ITS4. It was consisted of 38 bp of the 3' end of 18S rDNA, 174 bp of ITS1 region, 159 bp of 5.8S rDNA, 444 bp of the ITS2 region, and 46 bp of the 5' end of the 28S rDNA (results not shown).

Conventional PCR detection using species-specific primer

P. aphanidermatum ITS1 sequence in aliment against the ITS1 sequence of cucumber soil inhabiting pathogens showed *P. aphanidermatum* shares less than 35% identity. The candidate primer pair Paph54F-ITS2 designed for conventional PCR detection of *P. aphanidermatum* produced a clear amplicon of the expected size, a single band of about 200 bp using DNA from all *P. aphanidermatum* isolates as well as *P. aphanidermatum* infected cucumber stem tissue (Fig. 1B). As low as 200 fg pure genomic DNA could reliably be detected (Fig. 1A). The primer pair Paph54F-ITS2 were considered specific if no signal was obtained when using one ng of genomic DNA from all the separate nontarget organisms and uninfected cucumber stem tissue (results not shown).

DISCUSSION

This paper describes the successful identification of *P. aphanidermatum* based on morphological, molecular characteristics using specific conventional PCR primers for the DNA-mediated detection from infected cucumber tissue. Intensive vegetable cultivation in greenhouse could be increased soil borne plant pathogens such as *Fusarium*, *Verticillium*, and nematodes, in which spread rapidly, resulting in significant crop losses.

The genus *Pythium* is complex, containing more than 100 species. *P. aphanidermatum, P. irregulare, P. ultimum, P. pyrilabum,* and *P. middelltoni* are able to cause damping-off on cucumber and has been reported from different regions in Iran (Paul *et al.* 1996; Chehri *et al.* 2010; Hatami *et al.* 2010; Zamanizadeh *et al.* 2011). Our findings revealed the occurrence of *P. aphanidermatum* in commercially cucumber cultivation in Rafsanjan. No other inhabiting soil pathogens were identified. *P. aphanidermatum* is similar to *P. deliense* but oogonia in the latter pend toward antheridia wears the oogonial stalk is upright in the former. This character is not a clear criterion during identification. It is difficult and time consuming to distinguish these species using traditional morphological differences. For this reason, identification of *P. aphanidermatum* using molecular criteria is important. The GenBank-BLAST homology search using the ITS rDNA sequences of our isolates revealed *P. aphanidermatum* as the most similar sequence (>99% identity) with GenBank entries AB355599. We confirmed the identification of *P. aphanidermatum*, the causal agent of cucumber damping-off in Rafsanjan, using morphological and molecular identification.

A rapid, sensitive and specific detection method for this pathogen would be a powerful method for detecting or monitoring directly from plant material or in soil and water sample.

We have developed a rapid and reliable PCR-based DNA method to identify *P. aphanidermatum*. Candidate primer was designed based on sufficiently conserved parts of the rDNA ITS regions with attention to ITS sequence of *P. deliense* because it is morphologically similar to *P. aphanidermatum.* A pair oligonucleotid primer Paph54F-ITS2 selected for conventional PCR, which allowed amplification of a specific 200 bp DNA fragment of *P. aphanidermatum.* The sensitivity of conventional PCR was more than sufficient to confirm the pathogen's identity in symptomatic tissue. We obtained a detection limit of approximately 200 fg of pure *P. aphanidermatum* genomic DNA per PCR reaction, which is at least as good as reported for conventional PCR detection of other *Pythium* spp. (Kageyama *et al.* 1997; Wang & Chang 2003; Wang *et al.* 2003; Klemsdal *et al.* 2008). In specificity test either using BLAST-primer search or genomic DNA of closed species, no cross-reactions were observed with closed species. Similarly, no cross reactions were observed with genomic DNA from other soil-borne cucumber fungal pathogen or with any of the other fungi tested. The technique can be useful tools for detection and diagnosis of *P. aphanidermatum* in early stages of infection and help to reduce losses caused by this pathogen.

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